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14. ABSTRACT

Metastatic spread of prostate cancer is the second leading cause of deaths of men in theUnited States. Although there are many ways to treat non-metastatic form of prostate cancer ,only androgen-deprivation therapy is available for the extensive stage. Again, the cancerwill often progress to an androgen refractory (independent), metastatic stage. Recent reportshave suggested that the expression of VEGF-C and its receptor VEGFR-3 are directly correlatedwith lymph node dissemination in prostate cancer. This finding leads us to think thatunderstanding the role of angiogenic molecules like VEGF-C, -D in molecular detail forlymphatic formation in prostate cancer will provide us the information regarding theirrelationship with lymph node metastasis. We have observed significant increase in reactiveoxygen species and activation of small GTPase RalA upon androgen withdrawal, which in turnupregulates VEGF-C in prostate cancer cells. Interestingly our results suggest a function ofVEGF-C, which is directly related to its role in increasing the metastatic propensity ofprostate cancer rather than inducing lymphangiogenesis.

15. SUBJECT TERMS
vascular endothelial growth factor-C, lymphangiogenesis, metastasis

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Introduction: Metastatic spread of prostate cancer is the second leading cause of deaths of men in the United States. Every year almost 40.000 American men die from prostate cancer of which more than 70% die due to complications of late stage tumors that metastasize to distant location. Although there are many ways to treat non-metastatic form of prostate cancer like anti-androgen therapy, radical prostatectomy, radiotherapy and cytotherapy, only androgen-deprivation therapy is available for the extensive stage. Again, the cancer will often progress to an androgen refractory (independent), metastatic stage (1, 6, 17). However, the detailed molecular mechanism underlying the metastatic spread of this disease is poorly understood. Thus it has been difficult to develop effective treatments in this stage of prostate cancer. Recent reports have suggested that the expression of VEGF-C and its receptor VEGFR-3 are directly correlated with lymph node dissemination in prostate cancer (7, 16). This finding leads us to think that understanding the role of angiogenic molecules like VEGF-C, -D in molecular detail for lymphatic formation in prostate cancer will provide us the information regarding their relationship with lymph node metastasis. Thus, in this present study, the main focus will be to unravel the detail molecular mechanisms of these molecules that lead to the metastatic spread of prostate cancer to lymph node.

Body: Task1 described in the Statement of Work of our proposed application was as follows:

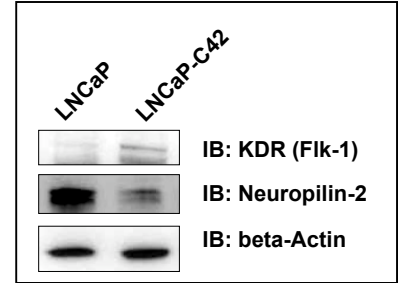*a. Develop a prostate cancer orthrotopic mouse model.*

We successfully accomplished the goal by generating the orthotopic human prostate cancer mouse models by implanting the LNCaP and PC3 cells. Detail of this model development was mentioned in our previous annual report.

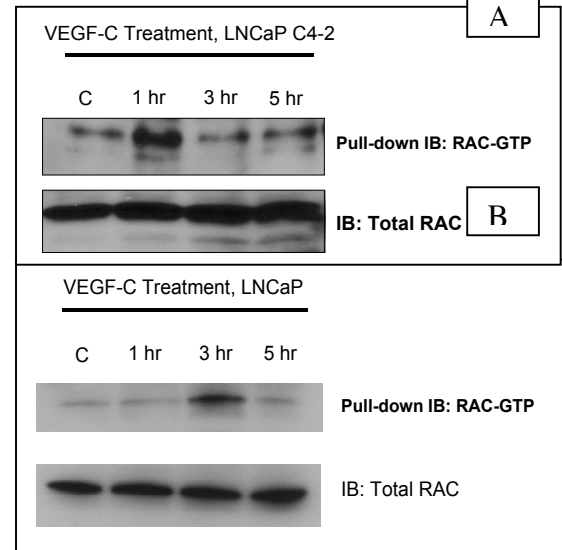b. *Detection of lymphangiogenesis in prostate cancer and its relation to lymph metastasis.*
The extent to which lymphangiogenesis is required for prostate cancer lymph node metastasis remains unclear in light of several contradictory reports published recently. It has been reported that peritumoral lymphatic vessel density correlates with lymph node metastasis in human prostate cancer (19). In another recent report Lin *et.al*. (9) suggested that VEGF-C-induced tumor-associated lymphangiogenesis was important for prostate cancer metastasis to the lymph node and lung. Inhibiting VEGF-C by overexpressing the soluble form of the VEGF-C receptor VEGFR3, inhibited both tumor-associated lymphangiogenesis and lymph node and lung metastasis. This study was carried out in a PC3-mIg2 (a variant of the PC3 cell line) xenograft mouse model. While these studies suggest the importance of lymphangiogenesis in prostate cancer lymph node metastasis, other reports are contradictory. One such report by Roma *et.al*. suggested that peritumoral lymphatic infiltration of tumor cell but not lymphangiogenesis was associated with regional lymph node metastases in prostate cancer (13). In another publication, Wong *et.al*. reported (18) that VEGF-C-induced lymphangiogenesis was unnecessary for lymph node metastasis in prostate cancer. With the inhibition of lymphangiogenesis in the PC3 xenograft mouse model, these authors did not detect any difference in metastasis. Therefore, more detailed studies are required to precisely determine the importance of lymphoangiogenesis in prostate cancer metastasis. In our previous annual report and also in our publication (8), we mentioned an increase in VEGF-C level during androgen ablated condition in prostate cancer cells indicating specific roles of VEGF-C during androgen refractory stage of the disease. In this light, the significance of lymphangiogenesis for promoting lymph node metastasis may not be crucial in the androgen refractory stage of prostate cancer, because systemic metastasis mainly occurs at this stage of the disease. **Several reports and our recent work now point to other VEGF-C functions that are independent of**

lymphangiogenesis but nevertheless important for tumor cell survival and metastasis. We believe that these functions for VEGF-C play significant roles in inducing tumor cell metastasis at the androgen refractory stage of prostate cancer. They are as follows:

- VEGF-C can stimulate the proliferation and migration of Kaposi's sarcoma cells (11) and also the proliferation and survival of leukemic cells (4).
- The lack of lymphangiogenesis observed in uveal melanoma, despite its high expression of VEGF-C (2).
- We have detected neuropilin-2 and VEGFR2, two of the known receptors of VEGF-C, in different prostate cancer cell lines, suggesting a possible autocrine function for VEGF-C (Figure 1).
- With VEGF-C stimulation, we have observed an increase in the expression of the androgen receptor co-activator, Bag-1L in LNCaP cells (8). Bag-1L has been reported to enhance the trans-activation function

Figure 1: Whole cell lysates from LNCaP and LNCaP-C42 cells were collected and resolved by SDS-PAGE. Western blot was performed using antibodies specific for KDR, Neuropilin-2 and β-actin (loading control).

of the androgen receptor with the help of Hsp70. Therefore; a VEGF-C-induced increase in Bag-1L indicates possible androgen receptor trans-activation even in the presence of low androgen concentrations, leading to the generation of a tumor with a more aggressive phenotype.

- Interestingly, we have observed an **increase in the level of GTP-bound Rac (functionally active Rac)** compared to total Rac protein in both androgen dependent (LNCaP) (Figure 2B) and androgen refractory (LNCaP C4-2) prostate cancer cells (Figure 2A) stimulated with VEGF-C. Interestingly, the kinetics of Rac activation upon VEGF-C treatment for androgen independent DU145 (data not shown) are more similar (activation occurs at 1 hour time point) to androgen refractory C4-2 cells than to androgen dependent LNCaP cells. Rac activation is associated with phyllopodia formation in the protruding edge of motile cell.

- We have observed a **decrease in the level of alpha-catenin in prostate cancer cells after VEGF-C stimulation (Figure 3)**. A decrease in alpha-catenin levels hinders E-cadherin and actin

Figure 2: (A) LNCaP-C42 and (B) LNCaP were cultured for 24 hours in serum starved conditions, and then stimulated with 200 ng/mL wild-type recombinant VEGF-C. The whole cell lysates were collected at different time points and subjected to pull-down assay for active Rac-GTP. Western blot was performed using antibody specific for Rac.

filament interaction and therefore disrupts cell-to-cell contact, which is required during cell migration.

- We have observed an **increase in the migration of androgen refractory LNCaP C4-2 cells** in a transwell cell migration assay with wild type recombinant VEGF-C stimulation (figure 4).

These results indicate that VEGF-C may increase the overall survival and the metastatic propensity of prostate tumor cells. **A similar autocrine loop has recently been described by Su *et. al.,* (14) who showed that VEGF-**

Figure 3: *LNCaP cells were cultured for 24 hours in serum conditions and then stimulated with 200 ng/mL wild-type recombinant VEGF-C for different time periods. Western blot analysis was performed using antibodies specific for α-E-catenin and β-actin internal loading control (upper panel). LNCaP cells cultured as described above were subjected to immunocytochemistry with antibody for α-E-catenin (lower panel).*

Immunocytochemistry of LNCaP cells with anti-alpha Catenin antibody
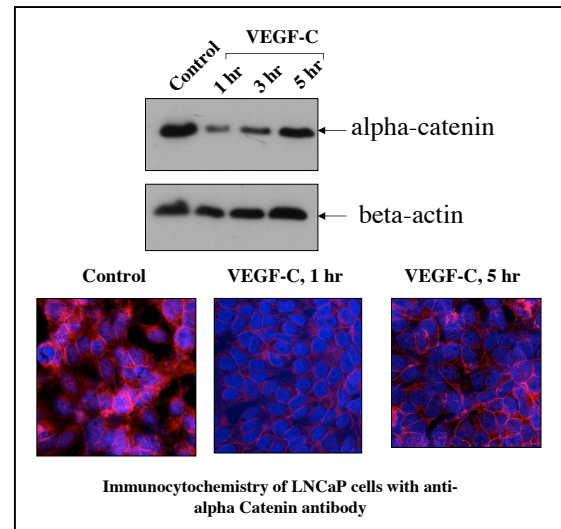
C promotes the invasion and metastasis of lung and breast tumors.

Taken together, our results and other reports suggest that VEGF-C may stimulate an increase in the metastatic propensity of prostate cancer cells, which would also

Figure 4: *LNCaP C4-2 cells were cultured in serum-starved conditions for 24 hours. 4.0 x 10^5 cells were placed in each upper chamber of an 8 micron pore transwell plate coated with 3 ug/mL human Vitronectin. Medium containing 200 ng/mL wild-type recombinant VEGF-C was used to stimulate migration. Migration assay was allowed to proceed for four hours before staining with Crystal Violet (Across).*

In Vitro Migration of LNCaP C4-2
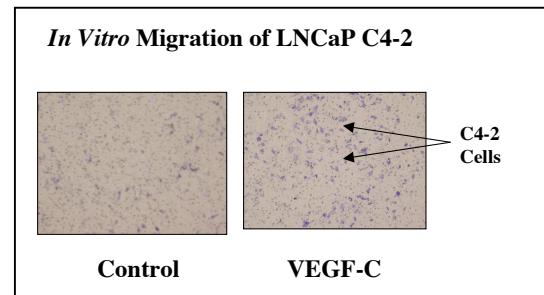
explain the observation that the peritumoral lymphatic infiltration of prostate cancer cells correlates with lymph node metastasis (19).

On the other hand, lymph node metastasis usually occurs in patients before the androgen refractory stage of the disease. Therefore, **the increase in the VEGF-C level during androgen withdrawal therapy might increase the survival of prostate cancer cells by increasing androgen receptor trans-activation (increasing Bag1L) during their transition to the androgen refractory stage and also help to enhance their systemic metastasis (increasing migratory property).** Based on this reasoning, we are also focusing on other functions of VEGF-C in promoting prostate cancer metastasis during androgen refractory stage other than lymhangiogenesis.

At present, we are working on to fulfill the following goals.

1. Preparation of stable LNCaP cell line that expresses Tet repressor:
2. Infection of pSUPERIOR into LNCaP-Tet cells
3. *In vivo* experiments for examining the effect of inhibition of the expression of VEGF-C and –D in prostate cancer.
One of the first steps to study the effect of VEGF-C and –D in prostate tumor growth in vivo is to detect the tumor growth in the mice in a non-invasive way. In order to achieve this goal, we have generated stable prostate cancer cell lines that will express luciferase gene. We implanted luciferase expressing tumor cell into the mouse prostate and was able to detect the tumor growth by detecting the luciferase activity. The detail of this model development has been described below.

Generation of orthotopic mouse tumor model with LNCaP C4-2 stably expressing Luciferase gene: We made stably expressing luciferase reporter LNCaP C4-2 cells using a lentivirus approach. The Luciferase expressing LNCaP C4-2 cells (~2X10^6 cells/50 µl medium) were injected orthotopically into male athymic BALB/c nude mice (NCI) (4-6 weeks old). Mice were anesthetized and tumors were imaged by IVIS 200 Imaging System (Xenogen) after 35 days of implantation. The mice at the left-hand side of figure 5a shows luciferase activity compare to the mice at the right suggesting the presence of the tumor. It was further confirmed in figure 5c, where tumor was detected by invasive way. Figure 5b shows different luciferase activity in three different mice suggesting different tumor sizes.

Figure 5: *Luciferase expressing LNCaP C4-2 cells were injected into the prostate of athymic mice. Tumors were imaged 35 days after implantation.*
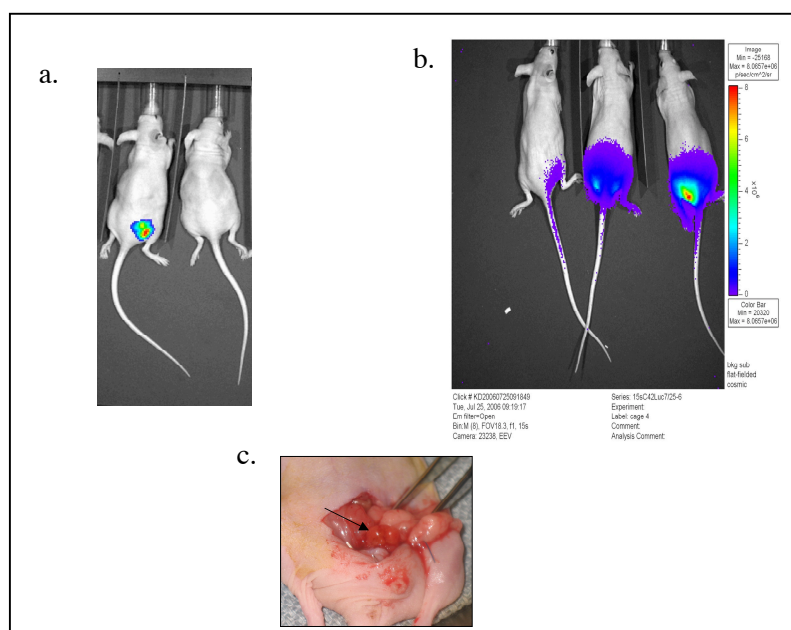
**Task 2. Elucidation of the molecular pathway that regulates the expression of VEGF-C in prostate cancer.**

Transcriptional regulation of VEGF-C in prostate cancer

Previously we have shown (described in detail in our previous Annual Report) that in the prostate cancer cell line LNCaP, VEGF-C up-regulation can also lead to an increase in androgen receptor co-activator, Bag-1L, suggesting that VEGF-C has the ability to transactivate androgen receptor under low androgen concentrations (8). Taken together, these findings suggest multiple functions for VEGF-C in prostate cancer progression. Despite this evidence for the role of VEGF-C in advanced stage prostate cancer, the molecular mechanism involved in VEGF-C expression is still poorly understood. In a previous publication, we show that androgen ablated conditions and a decrease in insulin-like growth factor 1-receptor (IGF-1R) signaling leads to activation of the forkhead transcription factor FOXO-1 and a concomitant upregulation of VEGF-C mRNA synthesis (8). However, the IGF-1R pathway for FOXO-1 activation is not the only pathway involved in the transcriptional regulation of VEGF-C under androgen ablated conditions. Recently, **we have reported in the journal *Oncogene* (2006, Sept 11, epub ahead of print) the involvement of the small GTPase RalA in the regulation of VEGF-C expression in prostate cancer.** The detail of these findings has been described below.

Androgen Deprivation results in increased intracellular ROS generation and VEGF-C synthesis: We have previously shown that in response to androgen deprivation, VEGF-C mRNA and protein levels are elevated (~3- 7 fold) in a time-dependent manner (8). Following our observation that androgen negatively regulates VEGF-C synthesis, publications describing androgen deprivation-induced upregulation of intracellular Reactive Oxygen Species (ROS) in rat prostate came to our attention (15). Based on these published reports, we were curious to analyze whether androgen deprivation could lead to increased ROS generation in the androgen-dependent human prostate carcinoma cell line LNCaP. To do so, we cultured LNCaP cells both in normal and charcoal-dextran (CS) treated (androgen deprived) serum for 72 hours, and then analyzed ROS production by flow cytometric detection as described in Methods. Under androgen depleted conditions, we observed a significant shift in the population positive for increased ROS production, as compared to that for cells cultured in normal serum (Figure 6C). We were also able to confirm this observation by fluorescence microscopy visualization of intracellular ROS (Figure 6A and B). These data suggest that androgen deprivation leads to increased ROS generation in the LNCaP prostate carcinoma cell line. In order to determine whether androgen deprivation-induced ROS generation is a signaling event involved in the regulation of VEGF-C mRNA level, we treated LNCaP cells cultured in CS serum with the generic ROS scavenger N-acetyl-cysteine (NAC). Total RNA was collected and subjected to real-time PCR for quantification of VEGF-C mRNA levels. In the presence of the NAC antioxidant, we observed a reduction in VEGF-C mRNA levels, as compared to those for LNCaP cells left untreated with NAC ($P<0.05$) (Figure 7A). LNCaP cells treated with NAC were also cultured in CS serum supplemented with synthetic androgen R1881 (10nM). As shown in Figure 7B, in the presence of synthetic androgen NAC did not show any further inhibition of VEGF-C mRNA level ($P<0.05$). This data suggests that elevation of intracellular ROS is dependent upon androgen deprivation and is a signaling event involved in the regulation of VEGF-C synthesis.
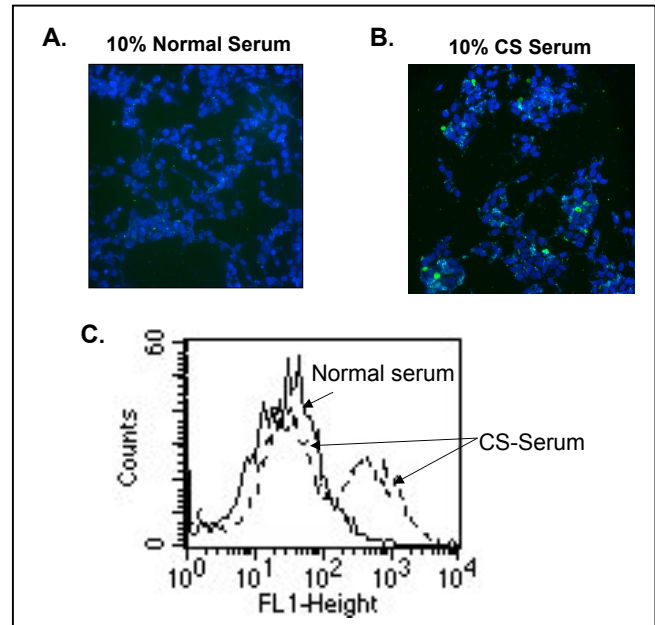
Figure 6 (A and B) LNCaP prostate carcinoma cells cultured in 10% normal and charcoal stripped (CS, androgen deprived) serum were subjected to reactive oxygen species (ROS) detection using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit, followed by flow cytometric detection (C) or fluorescence microscopy visualization.
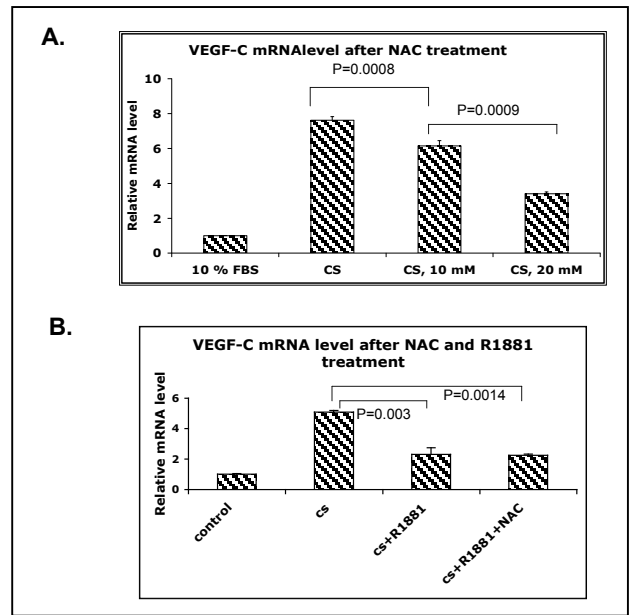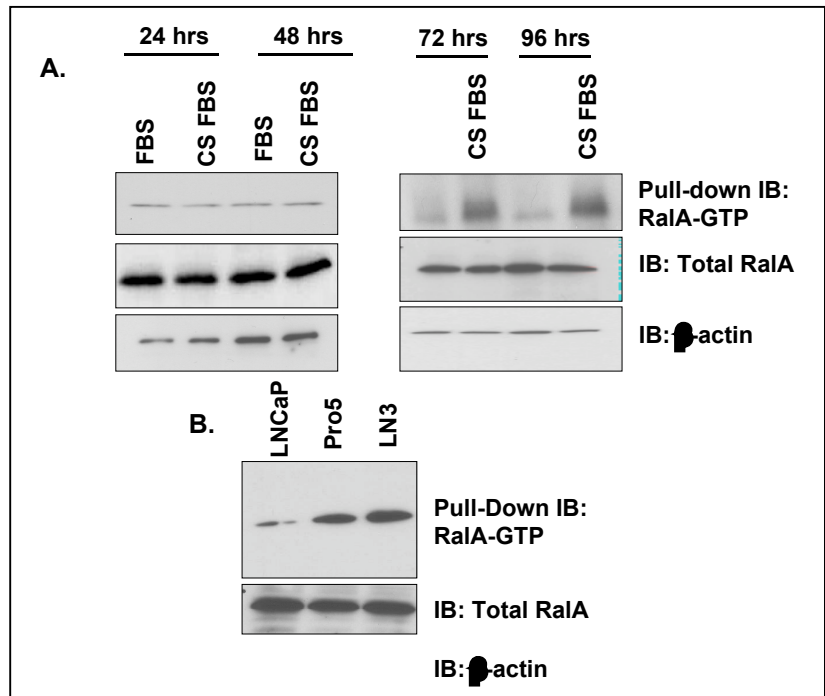
Ral A is activated under androgen ablated conditions: A report by Essers *et al.* has implicated the small GTPase RalA as a signaling molecule downstream of increased ROS generation (5). Recent reports implicating RalA in both human tumorigenic transformation and cancer metastasis led us to question whether RalA could be activated under conditions simulating androgen deprivation therapy, which is commonly used as treatment for advanced stage prostate cancer patients. As such, we chose to assess the involvement of RalA signaling as a downstream event for androgen-deprivation induced ROS generation. To do so, we cultured LNCaP cells under both normal and CS serum

Figure 7 (A) LNCaP cells cultured in 10% normal and CS serum for 72 hours were treated with 10 and 20 mM concentrations of NAC antioxidant. Total RNA was collected and subjected to real time PCR using primers for VEGF-C and 36B4. The data presented is the mean of 3 individual experiments. (B) LNCaP cells were cultured as above for 24 hours. The cell cultures were then supplemented with R1881 synthetic androgen (10 nM) for 48 hours, and then treated with the NAC antioxidant (20mM). Total RNA was collected and subjected to real time PCR using primers for VEGF-C and 36B4 internal control. All data presented is the mean of 3 individual experiments.

conditions for four different time points, and then performed a pull-down assay for activated RalA-GTP using Ral Binding Protein 1 (RalBP1) agarose as described in Methods. Protein eluted from the RalBP1 agarose was resolved by SDS-PAGE and immunoblotted with antibody against RalA. We found that under androgen deprived conditions, intracellular levels of active GTP-bound RalA were highly elevated at 72 and 96 hours, as compared to RalA-GTP levels for cells under normal serum conditions (Figure 8A). Both the 24 and 48-hour time points did not show significant upregulation of active RalA (Figure 8A). Total RalA levels were comparable between androgen deprived and non-androgen deprived cells as determined by

western blot. This finding suggests that RalA is activated within 72 hours of androgen deprivation in human prostate carcinoma cells. Of note, our previous findings (8) also support a significant upregulation of VEGF-C in LNCaP after 72 hours under androgen-
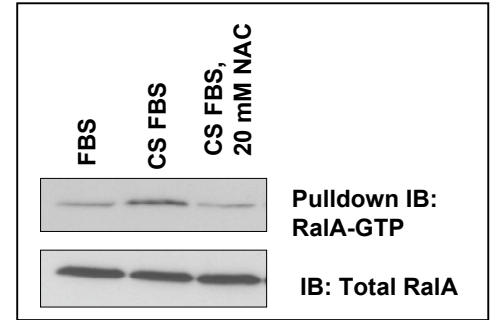
Figure 8 (A) LNCaP cells were cultured in 10% normal and CS serum for 24, 48, 72 and 96 hours. Cell lysate was subjected to a pull-down assay for activated RalA-GTP using RalBP1 agarose. Western blot was performed using antibodies for RalA and β-actin internal control. (B) LNCaP, LNCaP-Pro5 and LNCaP-LN3 cell lines were cultured in CS serum for 72 hours. Cell lysates were subjected to pull-down assay for activated RalA-GTP as above; western blot carried out using antibody for RalA and β-actin internal control.

ablated conditions. We have also observed a detectable increase in ROS level at 72 hours after androgen withdrawal (Figure 6). Therefore, taken together these results suggest that increased synthesis of VEGF-C, ROS and activation of RalA occur at similar time points.

In response to the observation that RalA is active under androgen-ablated conditions, we chose to compare active RalA-GTP levels across several prostate carcinoma cell lines. LNCaP and LNCaP syngenic cell lines LNCaP-LN3 and LNCaP-Pro5 were chosen as representative cell lines. The syngenic Pro5 and LN3 cell lines were originally generated by harvesting LNCaP tumor cells from either the prostate or lymph nodes of athymic mice. Pro5 and LN3 characteristically exhibit higher metastatic potential than the parental cell line (LNCaP < Pro5 < LN3) (12). Interestingly, an increase in the metastatic potential of these syngenic cells lines is also associated with a decrease in their growth requirement for androgen (12). Both Pro5 and LN3 were generously provided to us by Dr. Curtis A. Pettaway of MD Anderson. A pull-down immunoblot assay for activated RalA-GTP was performed with the cell lysates from LNCaP, Pro5, and LN3 as described. We observed that as compared to LNCaP, the more metastatic cell lines Pro5 and LN3 exhibited increased RalA activation (Figure 8B). In contrast, total RalA protein levels were comparable across all cell lines. This observation suggests that increasingly metastatic prostate carcinoma cell lines exhibit higher levels of RalA activation.
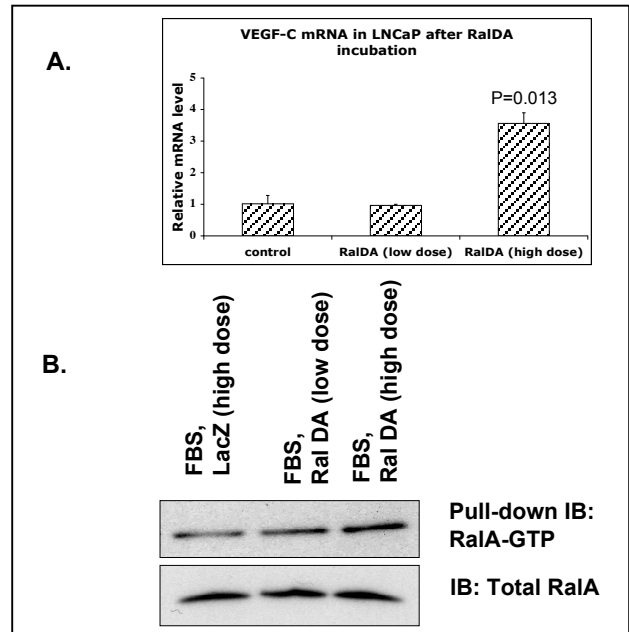

Figure 9. *LNCaP cells were cultured in 10% normal and CS serum for 72 hours. Cells cultured in CS serum were then treated with 20 mM NAC. Cell lysates were subjected to pull-down assay for active RalA-GTP as described. Western blot was carried out using antibody for RalA.*

Ral A activation is Reactive Oxygen Species (ROS) signaling dependent: Based on our previous finding that androgen deprivation increases intracellular ROS level, we chose to investigate whether RalA activation is directly regulated by ROS levels in our prostate cancer model. To do so, we cultured LNCaP cells in CS serum for 72 hours, and then treated these cells with generic ROS scavenger N-acetyl-cysteine (NAC) (20 mM). Intracellular levels of active (GTP-bound) RalA protein were measured using the pull-down immunoblot method as previously described. As expected, we observed that NAC treatment of LNCaP cells had an inhibitory effect on RalA activation (Figure 9).

VEGF-C is a downstream target of RalA signaling: Thus far our data suggested that androgen ablation of LNCaP prostate carcinoma cells leads to increased intracellular ROS generation and subsequent RalA activation. These findings prompted us to further investigate VEGF-C as a possible downstream target for RalA signaling. Using constitutively active RalA, we first chose to study whether VEGF-C mRNA level is regulated by RalA activation. We infected LNCaP cells cultured in normal serum with a retrovirus carrying the expression vector

for the constitutively active Ral Q75L (a generous gift from Dr. Chenning Der). Using real-time PCR, we observed an increase in the VEGF-C mRNA level of cells infected with Ral Q75L retrovirus, as compared to those infected with the LacZ control retrovirus (P<0.05) (Figure 10A). As expected, infection of LNCaP cells with the Ral Q75L retrovirus led to an increase in RalA-GTP levels, as compared to the control (Figure 10B). Using the RalN28 dominant negative expression vector kindly provided to us by Dr. Johannes L. Bos, we then studied whether VEGF-C mRNA level can
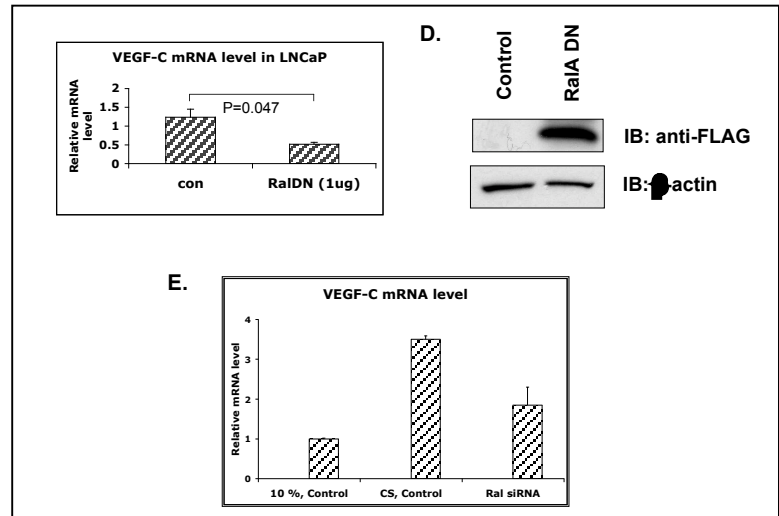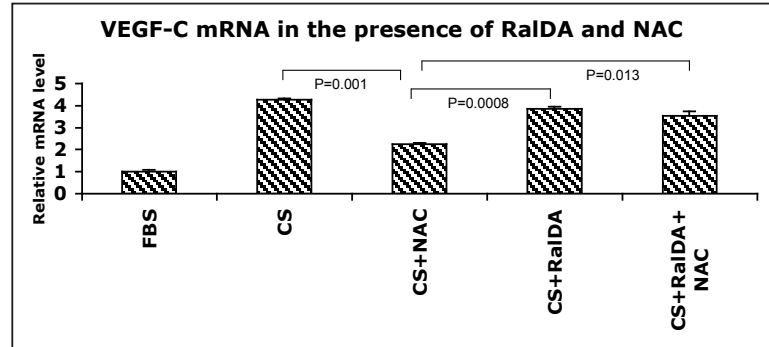

Figure 10 (A) LNCaP cells cultured in 10% normal serum were infected with low dose (0.4 ml) and high dose (0.8 ml) of RalA Q72L dominant active retrovirus or LacZ control retrovirus for 48 hours. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. The data presented here represents the mean of 3 individual experiments. (B) LNCaP cells were cultured in 10% FBS and infected with low (0.4 ml) and high (0.8 ml) dose of the RalA Q72L dominant active retrovirus and LacZ control retrovirus for 48 hours. Whole cell lysates were collected and subjected to pull-down assay for active RalA-GTP as described. Western blot was carried out using anti-RalA antibody. (C) LNCaP cells cultured in 10% normal and CS serum conditions were transfected with 1 μg of the RalA N28 dominant negative expression vector. After transfection for 48 hours, total RNA was collected and subjected to real time PCR using primers specific for VEGF-C and 36B4 internal control. The data presented represents the mean of 3 individual experiments. (D) LNCaP cells were transiently transfected with RalA N28 for 48 hours and cell lysates were collected and resolved using SDS-PAGE. Western blots with anti-FLAG tag and β-actin antibodies were carried out as previously described. (E) LNCaP cells cultured in 10% normal and CS serum were transfected with 100 nM RalA siRNA for 72 hours. Total RNA was collected and subjected to real time PCR using primers for VEGF-C and 36B4 internal control. The data presented represents the mean of 3 individual experiments.

be down-regulated by inhibiting RalA activation. RalN28 is a permanently GDP-bound, dominant negative form of RalA. LNCaP cells were cultured in CS serum for 24 hours and then transfected for 48 hours with the RalN28 dominant negative expression vector. Total RNA was collected and subjected to real-time PCR for quantification of VEGF-C mRNA levels. Upon transfection of LNCaP cells with the RalN28 dominant negative, we observed a statistically significant inhibition of VEGF-C mRNA level (Figure 10C). LNCaP cells transfected with the Ral N28 dominant negative expression vector showed significant expression of the vector as detected by western blot (Figure 10D). Additionally, cells transfected with the Ral N28 dominant negative and cultured in CS serum supplemented with synthetic androgen R1881 did not show any further inhibition of VEGF-C mRNA level (Figure 12B). We also performed a siRNA knockdown of RalA, which confirmed the inhibitory effect on VEGF-C mRNA level that we had previously observed with the Ral N28 dominant negative (P<0.05) (Figure 10E). These findings suggest that inhibition of RalA activity has an inhibitory effect on VEGF-C mRNA level. Taken

together, these findings confirm VEGF-C as a downstream target of RalA signaling, and suggest a positive correlation between RalA activation and VEGF-C synthesis.

Androgen ablation induces sequential signaling by ROS and RalA: Based on our previous observation that treatment of LNCaP cells with NAC antioxidant could inhibit VEGF-C mRNA level under androgen ablated conditions, we sought to confirm the possibility of a sequential ROS-RalA signaling axis. LNCaP cells cultured in CS (androgen ablated) serum were simultaneously infected with a Ral Q75L dominant active retrovirus and treated with the NAC antioxidant as described in
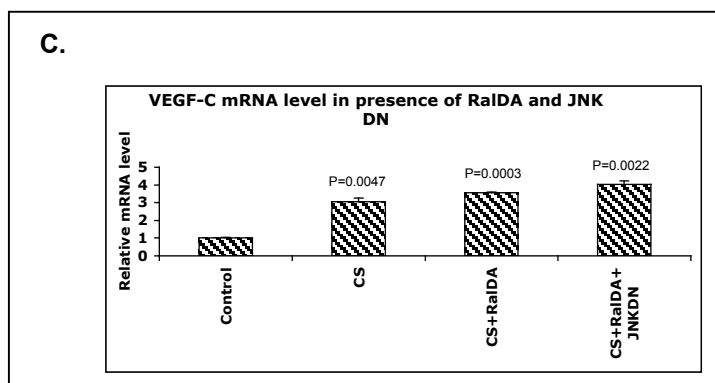

Methods. While treatment only with NAC inhibited VEGF-C mRNA level, infection with the RalA dominant active retrovirus restored VEGF-C mRNA levels in LNCaP cells treated with NAC (P<0.05) (Figure 11). Taken together, these

Figure 11. *LNCaP cells cultured in 10% normal and CS serum were infected with the Ral Q72L dominant active retrovirus for 48 hours and subsequently treated with NAC antioxidant (20mM). Total RNA was collected and subjected to real time PCR using primers for VEGF-C and 36B4 internal control.*

results suggest that increased ROS generation is an upstream signaling event for RalA activation in cells under androgen-ablated conditions.

JNK activation is not required for ROS/Ral A regulation of VEGF-C transcription: From previous reports, JNK appears to be a likely target for ROS-induced RalA signaling (3, 10). As such, we chose to study whether RalA stimulation of VEGF-C synthesis is mediated by JNK. LNCaP cells were cultured for 24 hours in normal and CS serum, and then transfected for 48 hours with either a control vector or the expression vector carrying the dominant negative form of JNK (JNK1) (kindly provided to us by Dr. Roger J. Davis of the University of Massachusetts). Total RNA was collected and VEGF-C mRNA levels were quantified by real-time PCR. Upon inhibition of JNK activity by the dominant negative, we observed a decrease in VEGF-C mRNA levels for LNCaP cells cultured in androgen deprived serum, as compared to those for LNCaP cells cultured in CS serum but transfected with the control vector (P<0.05) (Figure 12A). LNCaP cells were also transfected with the JNK1 dominant negative and cultured in CS serum supplemented with R1881. As expected, addition of androgen to the cell culture did not enhance the inhibitory effect of the JNK dominant negative on VEGF-C mRNA levels (Figure 12B). Taken together, these results suggest that JNK is
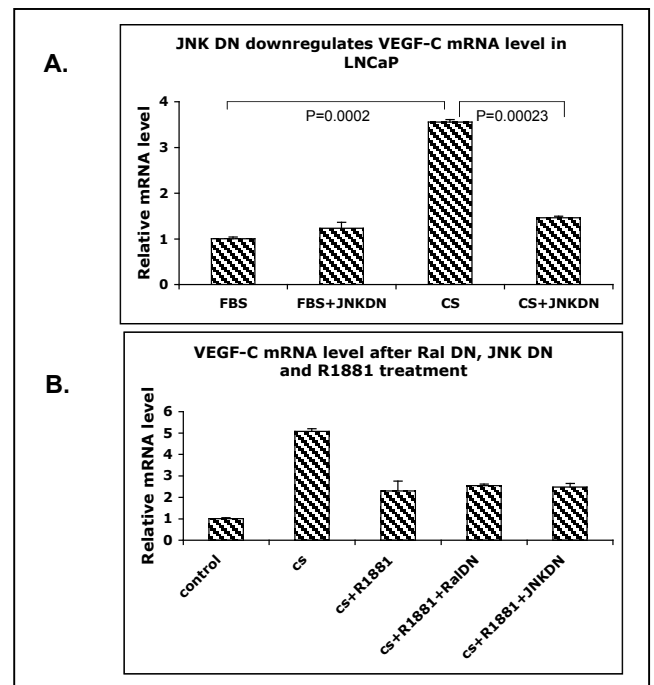
involved in upregulation of VEGF-C mRNA levels. Next, we chose to determine if JNK is in fact a downstream target of RalA signaling for VEGF-C upregulation upon androgen withdrawal. LNCaP cells were again cultured in both normal and CS serum for 24 hours, infected with retroviruses carrying the Ral Q75L dominant active and LacZ control vectors, and simultaneously transfected with the JNK-1 dominant negative expression vector for 48 hours. Real-time PCR quantification of VEGF-C mRNA levels showed that in cells infected with Ral Q75L

C.

VEGF-C mRNA level in presence of RalDA and JNK DN

Relative mRNA level

P=0.0047   P=0.0003   P=0.0022

Control   CS   CS+RalDA   CS+RalDA+ JNKDN

Figure 12 (A) LNCaP cells cultured in 10% normal and CS serum for 72 hours were transiently transfected with the JNK-1 dominant negative expression vector (1 μg) for 48 hours. Total RNA was collected and subjected to real time PCR using primers for VEGF-C and 36B4 internal control. The data presented is the mean of three individual experiments. (B) LNCaP cells were cultured in 10% normal and CS serum supplemented with 10 nM R1881. The cells were also transiently transfected with the dominant negative expression vectors for RalA and JNK for 48 hours. Total RNA was collected and real time PCR was performed using primers for VEGF-C and 36B4 internal control. The data presented is the mean of three individual experiments. (C) LNCaP cells were cultured as above, and simultaneously infected with RalA Q72L or LacZ control retroviruses and the JNK-1 dominant negative expression vector for 48 hours. Total RNA was collected and subjected to real time PCR using primers for VEGF-C and 36B4. The data presented is the mean of three individual experiments.

retrovirus, transfection with the dominant negative form of JNK did not inhibit VEGF-C mRNA levels under androgen ablated conditions ($P<0.05$) (Figure 12C). This result suggests that JNK activation may be involved in an additional pathway converging on VEGF-C synthesis, but that it does not seem to be involved for regulation of VEGF-C mRNA levels via RalA signaling.

Key Research Accomplishments:

1. We have successfully developed orthotopic mouse models of human prostate cancer that will enable us to study the role of VEGF-C in prostate cancer metastasis. We have also developed luciferase gene expressing prostate cancer cells that are helping us to image the tumor in the mouse in a non-invasive way.
2. We have observed functions of VEGF-C in increasing the metastatic propensity of prostate cancer cells. These functions of VEGF-C are distinct from its known function of inducing lymphangiogenesis.
3. We have delineated a molecular pathway, which involves the involvement of reactive oxygen species and small GTPase RalA for the transcriptional upregulation of VEGF-C in prostate cancer in the androgen-ablated condition.

Reportable Outcome:

Published paper:

Rinaldo F, Li J, Wang E, Muders M, **Datta K** RalA regulates vascular endothelial growth factor-C (VEGF-C) synthesis in prostate cancer cell during androgen ablation. *Oncogene* (2006, Sept 11, epub ahead of print)

Li J, Wang E, Rinaldo F, **Datta K**. (2005) Up-regulation of VEGF-C by androgen depletion: the involvement of IGF-IR-FOXO pathway. *Oncogene* 24(35), 5510-20.

Abstract presented: Li J, Wang E, Rinaldo F, **Datta K**. (2006) Regulation and Function of Vascular Endothelial Growth Factor-C in Prostate Cancer. *Prostate Spore Meeting*, Houston, Texas.
Li J, Wang E, Rinaldo F, **Datta K**. (2006) Regulation and Function of Vascular Endothelial Growth Factor-C in Prostate Cancer. *12th Spore Workshop*, Bultimore.

Plasmids generated: Generated LNCaP-C4-2-luciferase stable cell line, VEGF-C promoter luciferase expression construct in pGL3basic. siRNA of VEGF-C in pRetroSuper and pRetroSupirior.

Developed the orthotopic mouse model of human prostate cancer in immunocompromised mice. Developed stable luciferase and dsRed fluorescence protein expressing prostate cancer cells.

Conclusion:
1. In the first year of this grant, we were able to develop the orthotopic mouse model (mentioned in our 1st Annual report). Further progress has been made by creating stable cell lines that express luciferase gene and dsRed fluorescence protein and by studying the growth of prostate cancer in a non-invasive way.
2. Our studies indicated an interesting and distinct role of VEGF-C than its known function of Lymphangiogenesis in prostate cancer where it directly induces the metastatic potential of prostate cancer cells.
3. We have also delineated ROS and RalA mediated cellular events that upregulate VEGF-C in prostate cancer.

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ORIGINAL ARTICLE

RalA regulates vascular endothelial growth factor-C (VEGF-C) synthesis in prostate cancer cells during androgen ablation

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Prostate cancer mortality is primarily due to failure to cure patients with metastatic disease. In its early stages, prostate cancer growth is enhanced by androgens. As such, the primary therapy for advanced (locally extensive or metastatic) prostate cancer consists of androgen deprivation therapy by pharmacotherapeutic or surgical means. Eventually, the tumor recurs owing to a transition from androgen-dependence to a highly metastatic and androgen refractory (androgen depletion-independent) phenotype. As the detailed molecular mechanism underlying this transition to a more aggressive phenotype is poorly understood, it has been difficult to develop effective treatments for this advanced stage of the disease. We have previously reported an increase in vascular endothelial growth factor-C (VEGF-C) expression in human prostate cancer cells after androgen withdrawal. We have also shown increased expression of the androgen receptor co-activator BAG-1L by VEGF-C, suggesting the involvement of this growth factor in transactivation of the androgen receptor, even at low concentrations of androgen. In our present study, we show that androgen deprivation of human prostate carcinoma cells activates the small GTPase, RalA, a molecule important for human oncogenesis. RalA activation leads to VEGF-C upregulation. We also show that elevated levels of intracellular reactive oxygen species in prostate cancer cells under androgen-ablated conditions is the major inducer of RalA activation and VEGF-C synthesis.

Introduction

Prostate cancer is the second leading cause of cancer-related death for men in the United States. Advanced stage prostate cancer is usually treated with androgen deprivation therapy (American Cancer Society: Statistics for 2005). Although androgen deprivation therapy may modestly prolong survival, it is palliative and not curative. The vast majority of patients who initially respond to the therapy progress to the highly aggressive, androgen depletion-independent and highly metastatic stage of the disease (Kyprianou *et al.*, 1990; Denis and Murphy, 1993; Epstein *et al.*, 1996; Oh and Kantoff, 1998; Isaacs, 1999). It is acknowledged that vascular endothelial growth factor-C (VEGF-C) might play a role in the progression and metastatic spread of prostate tumors (Tsurusaki *et al.*, 1999; Jennbacken *et al.*, 2005; Zeng *et al.*, 2005). VEGF-C has a role in the lymphatic vessel growth and lymph node metastasis (Karpanen *et al.*, 2001; Mandriota *et al.*, 2001; Skobe *et al.*, 2001; He *et al.*, 2002). Other studies have shown a strong correlation between VEGF-C and its receptor VEGF-R3 (flt4) expression and lymph node metastasis in human prostate carcinoma tissue (Tsurusaki *et al.*, 1999; Jennbacken *et al.*, 2005; Zeng *et al.*, 2005). Previously, we have shown that in the prostate cancer cell line LNCaP, VEGF-C upregulation can also lead to an increase in androgen receptor co-activator, Bag-1L, suggesting that VEGF-C has the ability to transactivate androgen receptor under low androgen concentrations (Li *et al.*, 2005). Taken together, these findings suggest multiple functions for VEGF-C in prostate cancer progression. Despite this evidence for the role of VEGF-C in advanced stage prostate cancer, the molecular mechanism involved in VEGF-C expression is still poorly understood. In a previous publication, we show that androgen-ablated conditions and a decrease in insulin-like growth factor 1-receptor (IGF-1R) signaling leads to activation of the forkhead transcription factor FOXO-1 and a concomitant upregulation of VEGF-C mRNA synthesis (Li *et al.*, 2005). However, the IGF-1R pathway for FOXO-1 activation is not the only pathway involved in the transcriptional regulation of VEGF-C under androgen-ablated conditions. The present study demonstrates the involvement of the small GTPase RalA in the regulation of VEGF-C expression in prostate cancer.

Sequence similarity with H-, K- and N-Ras small G proteins first led to isolation of the Ras-like GTPase, RalA (Chardin and Tavitian, 1986), now considered a member of the Ras-family of GTPases. Ras family

G-proteins are considered to be critical effectors molecules for mediating a variety of cellular responses, including proliferation, differentiation and survival. Specifically, Ral GTPases RalA and RalB have been shown to serve essential functions in vesicle trafficking, cell morphology, transcriptional activation and human oncogenesis (Feig, 2003). Studies also suggest a role for RalA in tumor metastasis (Tchevkina *et al.*, 2005) and anchorage-independent proliferation (Chien and White, 2003). In contrast, activation of RalGEF and Ral Ras-effector proteins is not a potent inducer of transformation in rodent fibroblast or epithelial cells (Urano *et al.*, 1996; McFall *et al.*, 2001; Ulku *et al.*, 2003; Collette *et al.*, 2004). Taken together, these findings suggest that Ras-mediated oncogenesis occurs via distinct mechanisms in humans and mice, and highlight RalA and Ral-GEFS as unique potential targets for human cancer therapy.

A role for reactive oxygen species (ROS) in the pathogenesis of prostate cancer has also been well established. Most importantly, ROS may also induce signaling pathways involved in tumor cell survival under stress conditions. For instance, Tam *et al.* (2003) have shown that androgen deprivation leads to a significant increase in ROS production and the upregulation of ROS-generating nicotinamide adenine dinucleotide phosphate (reduced form) oxidases in rat acinar epithelial cells.

Recently, Essers *et al.* (2004) have confirmed the small GTPase RalA to be a downstream signaling molecule of ROS. They observed elevated intracellular levels of active or GTP-bound RalA in response to treatment with hydrogen peroxide, causing FOXO-4 nuclear translocation followed by transcriptional activation. These findings linking forkhead transcription factor activation with RalA activity, along with studies establishing a role for ROS and RalA in prostate tumor progression, led us to investigate a possible role for these signaling molecules in VEGF-C transcriptional regulation. Here, we describe a novel mechanism for VEGF-C transcriptional regulation in tumor-derived human prostate carcinoma cell lines.

Results

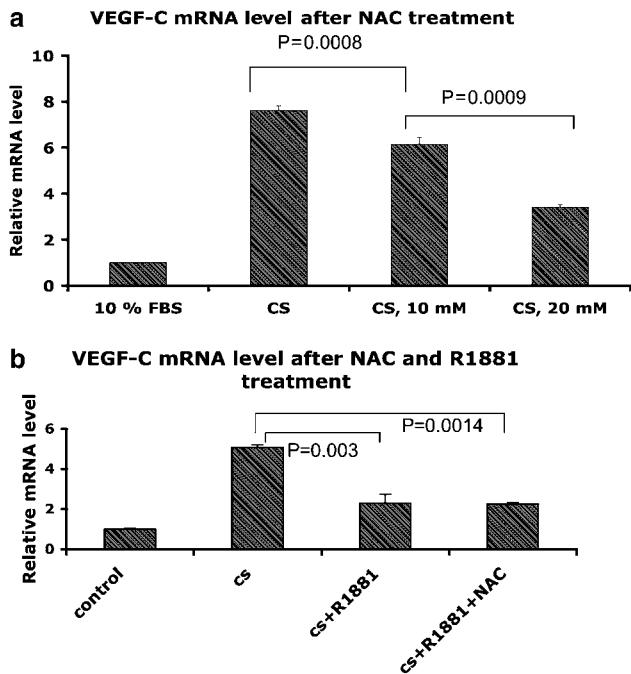### Androgen deprivation results in increased intracellular ROS generation and VEGF-C synthesis

We have previously shown that in response to androgen deprivation, VEGF-C mRNA and protein levels are elevated ($\sim$3- to 7-fold) in a time-dependent manner (Li *et al.*, 2005). Following our observation that androgen negatively regulates VEGF-C synthesis, publications describing androgen deprivation-induced upregulation of intracellular ROS in the rat prostate came to our attention (Tam *et al.*, 2003). Based on these published reports, we were curious to analyse whether androgen deprivation could lead to increased ROS generation in the androgen-dependent human prostate carcinoma cell line LNCaP. To do so, we cultured LNCaP cells both in normal and charcoal-dextran (charcoal stripped

(CS))-treated (androgen-deprived) serum for 72 h, and then analysed ROS production by flow cytometric detection as described in Materials and methods. Under androgen-depleted conditions, we observed a significant shift in the population positive for increased ROS production, as compared to that for cells cultured in normal serum (Figure 1c). We were also able to confirm this observation by fluorescence microscopy visualization of intracellular ROS (Figure 1a and b). These data suggest that androgen deprivation leads to increased ROS generation in the LNCaP prostate carcinoma cell line.

In order to determine whether androgen deprivation-induced ROS generation is a signaling event involved in the regulation of VEGF-C mRNA level, we treated LNCaP cells cultured in CS serum with the generic ROS scavenger *N*-acetyl-cysteine (NAC). Total RNA was collected and subjected to real-time polymerase chain reaction (PCR) for quantification of VEGF-C mRNA levels. In the presence of the NAC antioxidant, we observed a reduction in VEGF-C mRNA levels, as compared to those for LNCaP cells left untreated with NAC ($P < 0.05$) (Figure 2a). LNCaP cells treated with NAC were also cultured in CS serum supplemented with synthetic androgen R1881 (10 nM). As shown in Figure 2b, in the presence of synthetic androgen NAC did not show any further inhibition of VEGF-C mRNA level ($P < 0.05$). These data suggest that elevation of intracellular ROS is dependent upon androgen deprivation and is a signaling event involved in the regulation of VEGF-C synthesis.
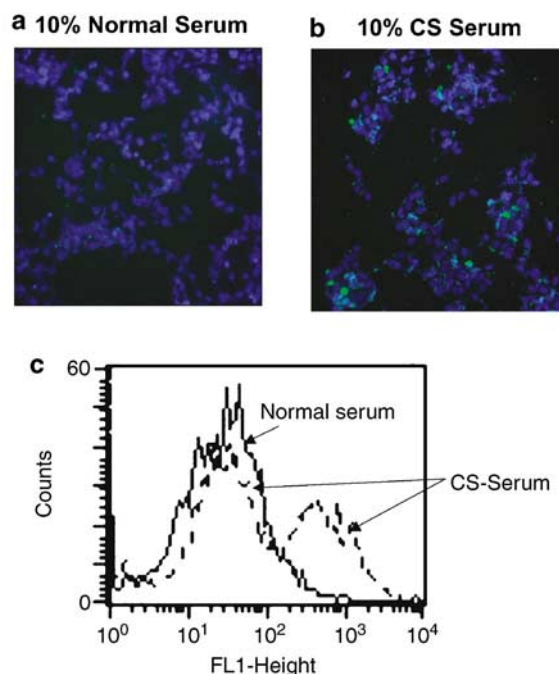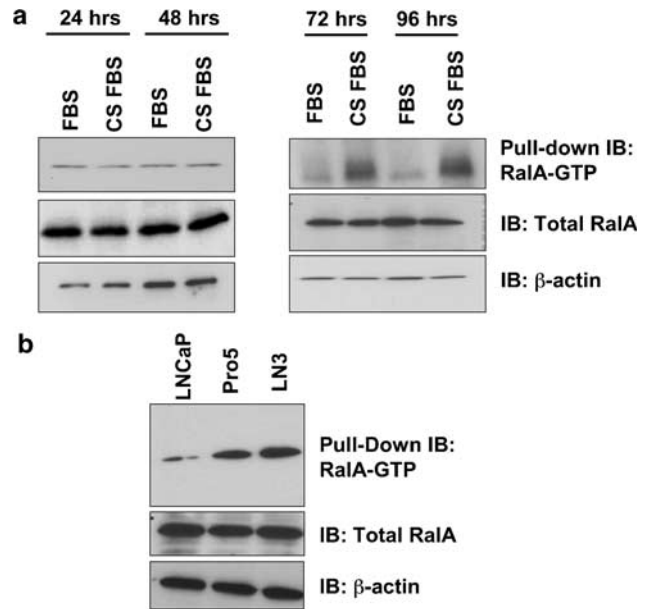

Figure 1 (**a** and **b**) LNCaP prostate carcinoma cells cultured in 10% normal and charcoal-stripped (CS, androgen-deprived) serum were subjected to ROS detection using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit, followed by flow cytometric detection (**c**) or fluorescence microscopy visualization.

a

VEGF-C mRNA level after NAC treatment

P=0.0008

P=0.0009

10 % FBS   CS   CS, 10 mM   CS, 20 mM

b

VEGF-C mRNA level after NAC and R1881 treatment

P=0.003

P=0.0014

control   cs   cs+R1881   cs+R1881+NAC

Figure 2 (a) LNCaP cells cultured in 10% normal and CS for 72 h were treated with 10 and 20 mM concentrations of NAC antioxidant. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4. The data presented are the mean of three individual experiments. (b) LNCaP cells were cultured as above for 24 h. The cell cultures were then supplemented with R1881 synthetic androgen (10 nM) for 48 h, and then treated with the NAC antioxidant (20 mM). Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. All data presented are the mean of three individual experiments.

a

24 hrs   48 hrs   72 hrs   96 hrs

Pull-down IB: RalA-GTP

IB: Total RalA

IB: β-actin

b

LNCaP   Pro5   LN3

Pull-Down IB: RalA-GTP

IB: Total RalA

IB: β-actin

Figure 3 (a) LNCaP cells were cultured in 10% normal and CS for 24, 48, 72 and 96 h. Cell lysate was subjected to a pull-down assay for activated RalA-GTP using RalBP1 agarose. Western blot was performed using antibodies for RalA and β-actin internal control. (b) LNCaP, LNCaP-Pro5 and LNCaP-LN3 cell lines were cultured in CS for 72 h. Cell lysates were subjected to pull-down assay for activated RalA-GTP as above; Western blot carried out using antibody for RalA and β-actin internal control.

RalA is activated under androgen-ablated conditions

A report by Essers et al. (2004) has implicated the small GTPase RalA as a signaling molecule downstream of increased ROS generation. Recent reports implicating RalA in both human tumorigenic transformation and cancer metastasis led us to question whether RalA could be activated under conditions simulating androgen deprivation therapy, which is commonly used as treatment for advanced stage prostate cancer patients. As such, we chose to assess the involvement of RalA signaling as a downstream event for androgen deprivation-induced ROS generation. To do so, we cultured LNCaP cells under both normal and CS conditions for four different time points, and then performed a pull-down assay for activated RalA-GTP using Ral binding protein 1 (RalBP1) agarose as described in Materials and methods. Protein eluted from the RalBP1 agarose was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with antibody against RalA. We found that under androgen-deprived conditions, intracellular levels of active GTP-bound RalA were highly elevated at 72 and 96 h, as compared to RalA-GTP levels for cells under normal serum conditions (Figure 3a). Both the 24- and 48-h time points did not show significant

upregulation of active RalA (Figure 3a). Total RalA levels were comparable between androgen-deprived and non-androgen-deprived cells as determined by Western blot. This finding suggests that RalA is activated within 72 h of androgen deprivation in human prostate carcinoma cells. Of note, our previous findings (Li et al., 2005) also support a significant upregulation of VEGF-C in LNCaP after 72 h under androgen-ablated conditions. We have also observed a detectable increase in ROS level at 72 h after androgen withdrawal (Figure 1). Therefore, taken together, these results suggest that increased synthesis of VEGF-C, ROS and activation of RalA occur at similar time points.

In response to the observation that RalA is active under androgen-ablated conditions, we chose to compare active RalA-GTP levels across several prostate carcinoma cell lines. LNCaP and LNCaP syngenic cell lines LNCaP-LN3 and LNCaP-Pro5 were chosen as representative cell lines. The syngenic Pro5 and LN3 cell lines were originally generated by harvesting LNCaP tumor cells from either the prostate or lymph nodes of athymic mice. Pro5 and LN3 characteristically exhibit higher metastatic potential than the parental cell line (LNCaP < Pro5 < LN3) (Pettaway et al., 1996). Interestingly, an increase in the metastatic potential of these syngenic cells lines is also associated with a decrease in their growth requirement for androgen (Pettaway et al., 1996). Both Pro5 and LN3 were generously provided to us by Dr Curtis A Pettaway of MD Anderson. A pull-down immunoblot assay for activated RalA-GTP was

performed with the cell lysates from LNCaP, Pro5 and LN3 as described. We observed that as compared to LNCaP, the more metastatic cell lines Pro5 and LN3 exhibited increased RalA activation (Figure 3b). In contrast, total RalA protein levels were comparable across all cell lines. This observation suggests that increasingly metastatic prostate carcinoma cell lines exhibit higher levels of RalA activation.

RalA activation is ROS signaling-dependent
Based on our previous finding that androgen deprivation increases intracellular ROS level, we chose to investigate whether RalA activation is directly regulated by ROS levels in our prostate cancer model. To do so, we cultured LNCaP cells in CS for 72 h, and then treated these cells with generic ROS scavenger NAC (20 mM). Intracellular levels of active (GTP-bound) RalA protein were measured using the pull-down immunoblot method as described previously. As expected, we observed that NAC treatment of LNCaP cells had an inhibitory effect on RalA activation (Figure 4).

VEGF-C is a downstream target of RalA signaling
Thus far, our data suggested that androgen ablation of LNCaP prostate carcinoma cells leads to increased intracellular ROS generation and subsequent RalA activation. These findings prompted us to further investigate VEGF-C as a possible downstream target for RalA signaling. Using constitutively active RalA, we first chose to study whether VEGF-C mRNA level is regulated by RalA activation. We infected LNCaP cells cultured in normal serum with a retrovirus carrying the expression vector for the constitutively active Ral Q75L (a generous gift from Dr Chenning Der). Using real-time PCR, we observed an increase in the VEGF-C mRNA level of cells infected with Ral Q75L retrovirus, as compared to those infected with the LacZ control retrovirus ($P<0.05$) (Figure 5a). As expected, infection of LNCaP cells with the Ral Q75L retrovirus led to an increase in RalA-GTP levels, as compared to the control (Figure 5b). Using the RalN28 dominant-negative expression vector kindly provided to us by Dr Johannes L Bos, we then studied whether VEGF-C mRNA level can be downregulated by inhibiting RalA activation. RalN28 is a permanently GDP-bound,

dominant-negative form of RalA. LNCaP cells were cultured in CS for 24 h and then transfected for 48 h with the RalN28 dominant-negative expression vector. Total RNA was collected and subjected to real-time PCR for quantification of VEGF-C mRNA levels. Upon transfection of LNCaP cells with the RalN28 dominant-negative, we observed a statistically significant inhibition of VEGF-C mRNA level (Figure 5c). LNCaP cells transfected with the RalN28 dominant-negative expression vector showed significant expression of the vector as detected by Western blot (Figure 5d). Additionally, cells transfected with the RalN28 dominant-negative and cultured in CS supplemented with synthetic androgen R1881 did not show any further inhibition of VEGF-C mRNA level (Figure 7b). We also performed a small interfering RNA knockdown of RalA, which confirmed the inhibitory effect on VEGF-C mRNA level that we had previously observed with the RalN28 dominant-negative ($P<0.05$) (Figure 5e) (see Supplement). These findings suggest that inhibition of RalA activity has an inhibitory effect on VEGF-C mRNA level. Taken together, these findings confirm VEGF-C as a downstream target of RalA signaling, and suggest a positive correlation between RalA activation and VEGF-C synthesis.

*Androgen ablation induces sequential signaling by ROS and RalA*
Based on our previous observation that treatment of LNCaP cells with NAC antioxidant could inhibit VEGF-C mRNA level under androgen-ablated conditions, we sought to confirm the possibility of a sequential ROS–RalA signaling axis. LNCaP cells cultured in CS (androgen ablated) serum were simultaneously infected with a Ral Q75L dominant-active retrovirus and treated with the NAC antioxidant as described in Materials and methods. Although treatment with NAC inhibited VEGF-C mRNA level, infection with the RalA dominant-active retrovirus restored VEGF-C mRNA levels in LNCaP cells treated with NAC ($P<0.05$) (Figure 6). Taken together, these results suggest that increased ROS generation is an upstream signaling event for RalA activation in cells under androgen-ablated conditions.

*JNK activation is not required for ROS/RalA regulation of VEGF-C transcription*
From previous reports, c-Jun N-terminal kinase (JNK) appears to be a likely target for ROS-induced RalA signaling (Lo et al., 1996; de Ruiter et al., 2000). As such, we chose to study whether RalA stimulation of VEGF-C synthesis is mediated by JNK. LNCaP cells were cultured for 24 h in normal and CS, and then transfected for 48 h with either a control vector or the expression vector carrying the dominant-negative form of JNK (JNK1) (kindly provided to us by Dr Roger J Davis of the University of Massachusetts). Total RNA was collected and VEGF-C mRNA levels were quantified by real-time PCR. Upon inhibition of JNK activity by the dominant-negative, we observed a decrease in
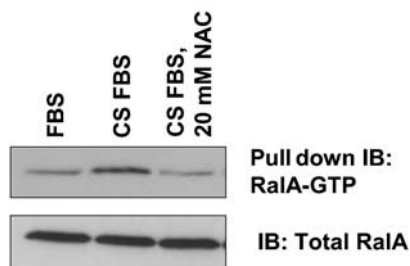
Figure 4 LNCaP cells were cultured in 10% normal and CS for 72 h. Cells cultured in CS were then treated with 20 mM NAC. Cell lysates were subjected to pull-down assay for active RalA-GTP as described. Western blot was carried out using antibody for RalA.
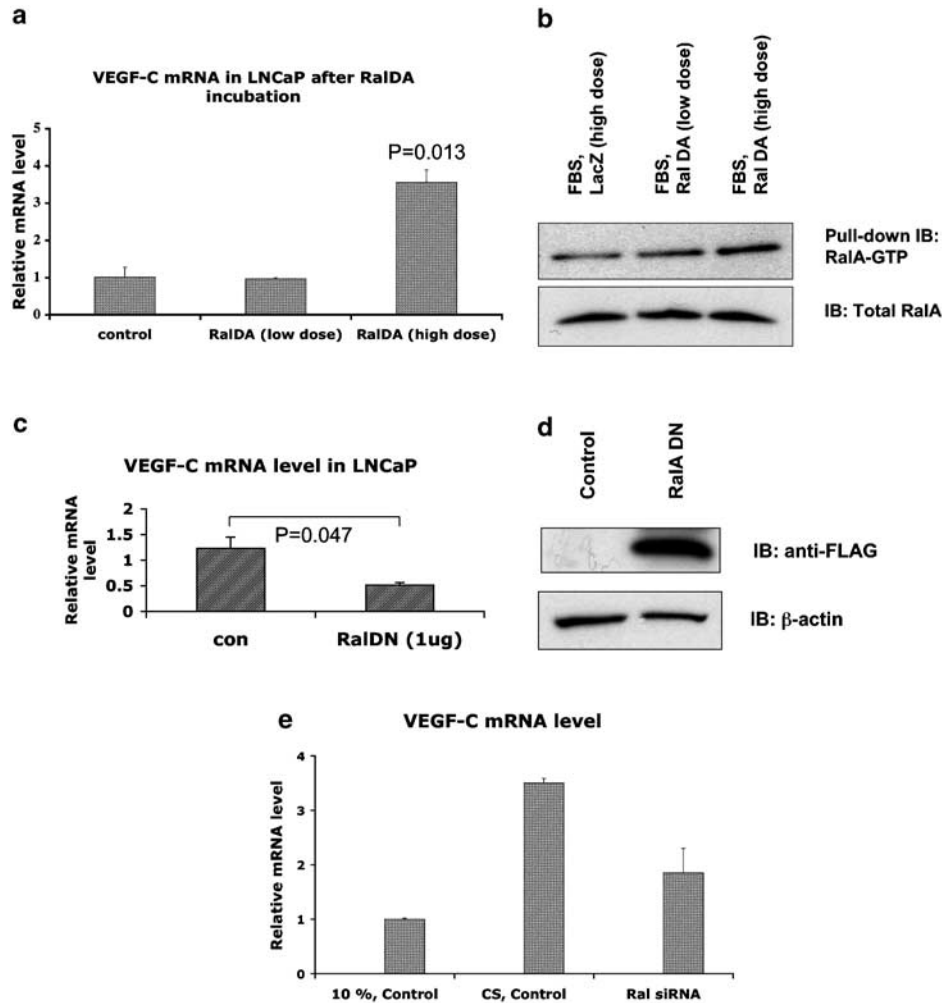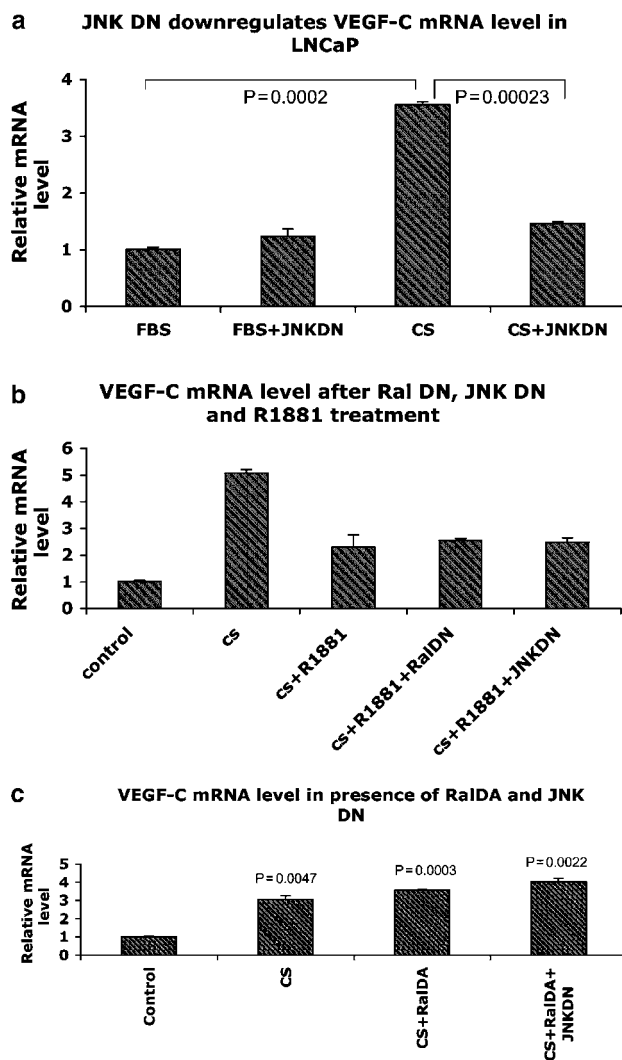
Figure 5 (a) LNCaP cells cultured in 10% normal serum were infected with low dose (0.4 ml) and high dose (0.8 ml) of RalA Q72L dominant-active retrovirus or LacZ control retrovirus for 48 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. The data presented here represent the mean of three individual experiments. (b) LNCaP cells were cultured in 10% FBS and infected with low (0.4 ml) and high (0.8 ml) dose of the RalA Q72L dominant-active retrovirus and LacZ control retrovirus for 48 h. Whole-cell lysates were collected and subjected to pull-down assay for active RalA-GTP as described. Western blot was carried out using anti-RalA antibody. (c) LNCaP cells cultured in 10% normal and CS conditions were transfected with 1 μg of the RalA N28 dominant-negative expression vector. After transfection for 48 h, total RNA was collected and subjected to real-time PCR using primers specific for VEGF-C and 36B4 internal control. The data presented represent the mean of three individual experiments. (d) LNCaP cells were transiently transfected with RalA N28 for 48 h and cell lysates were collected and resolved using SDS–PAGE. Western blots with anti-FLAG tag and β-actin antibodies were carried out as described previously. (e) LNCaP cells cultured in 10% normal and CS were transfected with 100 nM RalA small interfering RNA for 72 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. The data presented represent the mean of three individual experiments.
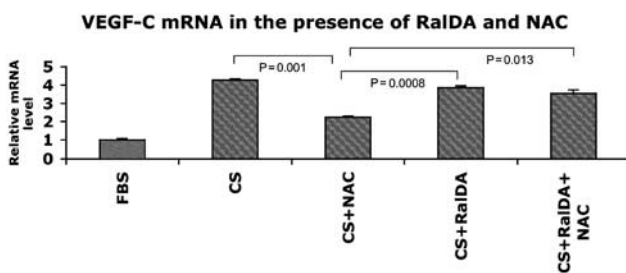
Figure 6 LNCaP cells cultured in 10% normal and CS were infected with the Ral Q72L dominant-active retrovirus for 48 h and subsequently treated with NAC antioxidant (20 mM). Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control.

VEGF-C mRNA levels for LNCaP cells cultured in androgen-deprived serum, as compared to those for LNCaP cells cultured in CS but transfected with the control vector ($P < 0.05$) (Figure 7a). LNCaP cells were also transfected with the JNK1 dominant-negative and cultured in CS supplemented with R1881. As expected, addition of androgen to the cell culture did not enhance the inhibitory effect of the JNK dominant-negative on VEGF-C mRNA levels (Figure 7b). Taken together, these results suggest that JNK is involved in the upregulation of VEGF-C mRNA levels. Next, we chose to determine if JNK is in fact a downstream target of RalA signaling for VEGF-C upregulation upon

a JNK DN downregulates VEGF-C mRNA level in LNCaP

b VEGF-C mRNA level after Ral DN, JNK DN and R1881 treatment

c VEGF-C mRNA level in presence of RalDA and JNK DN

Figure 7 (**a**) LNCaP cells cultured in 10% normal and CS for 72 h were transiently transfected with the JNK-1 dominant-negative expression vector (1 μg) for 48 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. The data presented are the mean of three individual experiments. (**b**) LNCaP cells were cultured in 10% normal and CS supplemented with 10 nM R1881. The cells were also transiently transfected with the dominant-negative expression vectors for RalA and JNK for 48 h. Total RNA was collected and real-time PCR was performed using primers for VEGF-C and 36B4 internal control. The data presented are the mean of three individual experiments. (**c**) LNCaP cells were cultured as above, and simultaneously infected with RalA Q72L or LacZ control retroviruses and the JNK-1 dominant-negative expression vector for 48 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4. The data presented are the mean of three individual experiments.

androgen withdrawal. LNCaP cells were again cultured in both normal and CS for 24 h, infected with retroviruses carrying the Ral Q75L dominant-active and LacZ control vectors, and simultaneously transfected with the JNK-1 dominant-negative expression vector for 48 h. Real-time PCR quantification of VEGF-C mRNA levels showed that in cells infected with Ral Q75L retrovirus, transfection with the dominant-negative

form of JNK did not inhibit VEGF-C mRNA levels under androgen-ablated conditions ($P < 0.05$) (Figure 7c). This result suggests that JNK activation may be involved in an additional pathway converging on VEGF-C synthesis, but that it does not seem to be involved for regulation of VEGF-C mRNA levels via RalA signaling.

Discussion

Several cancers show evidence of increased VEGF-C expression during their development and progression (Kimura et al., 2003; Neuchrist et al., 2003; Jia et al., 2004; Sipos et al., 2004). Elevated VEGF-C protein levels have also been observed in human prostate cancer specimens (Tsurusaki et al., 1999; Jennbacken et al., 2005). As the molecular pathways contributing to the androgen refractory, highly metastatic stage of prostate cancer are poorly understood, the potential involvement of VEGF-C in the transition to the androgen depletion-independent phenotype of prostate cancer is an important subject area for intense research. Our previous report provided evidence for the increased expression of VEGF-C under androgen-depleted conditions, suggesting a role for this growth factor in promoting the survival of prostate tumor cells during androgen withdrawal (Li et al., 2005). Based on these findings, it is likely that VEGF-C expression is a critical signal involved in prostate cancer progression to the androgen depletion-independent stage. The present study sought to elucidate one of several molecular pathways leading to the high expression of VEGF-C in prostate cancer under androgen-deprived conditions.

An increase in the generation of intracellular ROS owing to androgen withdrawal has been previously reported in rat acinar epithelial cells (Tam et al., 2003). Here, we show that a similar increase in ROS generation also occurs in prostate cancer cells during androgen withdrawal. As a result of high intracellular levels of ROS, there may be several downstream signaling events contributing to tumorigenesis. One of them, as we observed, is RalA activation leading to VEGF-C production. Activation of RalA has been shown to be important for human cancer progression (Feig, 2003; Tchevkina et al., 2005). In this light, activation of RalA under androgen-ablated conditions is in itself an important observation, suggesting RalA's possible involvement in the transition of prostate cancer towards the androgen refractory phenotype.

Based on our previous observation that VEGF-C is highly expressed under androgen-ablated conditions (Li et al., 2005), we questioned whether RalA activation might also regulate VEGF-C expression at low levels of androgen. Our finding that RalA can regulate VEGF-C expression provides an alternative mechanism for regulation of VEGF-C that is distinctly different from that of IGF-IR signaling. To our knowledge, this is the first report suggesting that activation of RalA, an event known for its importance in human oncogenesis, is induced during androgen ablation and leads to VEGF-C

synthesis. JNK activation is a known downstream signaling event of ROS (de Ruiter et al., 2000). Our studies point out that JNK can regulate VEGF-C synthesis. Of note, although RalA can activate JNK in other cell types (Essers et al., 2004), our study suggests that in prostate cancer cells JNK is not the downstream target of RalA for VEGF-C synthesis.

Individually, ROS generation, activation of RalA and increased expression of VEGF-C have been reported by other studies to lead to cancer progression and metastasis by several known mechanisms. In the present study, we not only show that these potent tumor inducers are activated owing to androgen withdrawal in prostate cancer, but also that they are components in a unified, sequential signaling pathway for the production of an important angiogenic growth factor, VEGF-C. Taken together, our findings suggest the existence of a ROS-RalA-VEGF-C signaling axis leading to enhanced VEGF-C synthesis in prostate cancer cells – that may be important for the transition of prostate cancer from androgen dependence to the androgen refractory stage. Hypoxia is one of the strong modulators of VEGF-A synthesis in both physiological and pathological conditions. Interestingly, VEGF-C is not induced by hypoxia. Until now, very little is known about how VEGF-C expression is regulated in cancer cells. Our study is therefore significant because it elucidates a hypoxia-independent molecular pathway involved in induction of VEGF-C production, and thus paves the way for future therapeutic intervention in the treatment of advanced stage prostate cancer.

Materials and methods

Cell culture
Human prostate cancer cell line LNCaP (ATCC # CRL-1740),) and LNCaP syngenic cell lines LNCaP-Pro5 and LNCaP-LN3 were cultured at 37°C in Rosewell Park Memorial Institute medium (RPMI) 1640 with L-glutamine (Mediatech Inc., Herndon, VA, USA) supplemented with penicillin/streptomycin and containing either 10% normal fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) or 10% charcoal-stripped (CS, androgen-depleted) FBS (Biomeda Corp., Foster City, CA, USA).

ROS detection and ROS scavenger treatment
LNCaP cells were grown in 60 mm cell culture dishes in RPMI containing either 10% normal or CS (androgen-depleted) serum for 72 h. For fluorescence microscopy visualization, cells were grown on glass coverslips placed inside the 60 mm cell culture dishes and then fixed in 4% formaldehyde phosphate-buffered saline. ROS detection was achieved using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit I36007 (Invitrogen Molecular Probes, Carlesbad, CA, USA), followed by either flow cytometric detection or fluorescence microscopy

visualization. For treatment with ROS scavenger, LNCaP cells were cultured in RPMI containing 10% normal or CS FBS for 72 h. The cells were then incubated with 20 mM N-acetyl-L-cysteine (NAC) ROS scavenger reagent (Sigma-Aldrich, Saint Louis, MO, USA) at 37.4°C for 2 h. Incubation with NAC was followed by total RNA isolation and VEGF-C mRNA quantification by real-time PCR as described below.

Active RalA detection assay
LNCaP, LNCaP-Pro5 and LNCaP-LN3 cells were grown in the presence or absence of androgen for 72 and 96 h, lysed with ice-cold radioimmunoprecipitation assay lysis buffer, incubated on ice for 10 min, and centrifuged at 10 000 r.p.m., 4°C for 10 min. The pull-down assays were performed using 0.5 mg of cellular protein from whole-cell extracts and 30 μl 50% slurry RalBP1 agarose (Upstate Biotechnology, Lake Placid, NY, USA).

Western blot analysis
The whole-cell extracts were separated by SDS–PAGE; immunodetection antibodies against RalA (Upstate Biotechnology, Lake Placid, NY, USA) and β-actin (Sigma-Aldrich) were used, followed by the secondary antibody incubation and detection by Enhanced Chemiluminescent (ESL) Substrate reagent from Amersham Biosciences Corp (Piscataway, NJ, USA).

RNA isolation and real-time PCR
RNA was isolated from LNCaP cells according to the RNeasy Minikit protocol for animal cells (Qiagen Inc., Valencia, CA, USA). The sequences for human VEGF-C and human 36B4 (housekeeping gene) were obtained from the PubMed Gene Bank and synthesized (Integrated DNA Technologies, Coralville, IA, USA) and primers were designed for real-time PCR. VEGF-C: forward, 5′-AGG GTC AGG CAG CGA ACA AGA-3′. VEGF-C: reverse, CCT CCT GAG CCA GGC ATC TG-3′. VEGF-C: middle, 5′-TGC CCC ACC AAT TAC ATG TGG AAT AAT CA-3′. 36B4: forward, 5′-ATA CAG CAG ATC CGC ATG T-3′. 36B4: reverse, 5′-TCA TGG TGT TCT TGC CCA TCA-3′. 36B4: middle, 5′-CAC CAC AGC CTT CCC GCG AA-3′. Real-time PCR was performed according to the Taqman method (see Supplement).

Statistics
For comparison between individual groups, t-tests with the assumptions of a two-tail distribution and two samples with equal variance were performed. A P-value below 0.05 was considered significant.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).